AD__________________
(Leave blank)

Award Number: W81XWH-07-1-0587

TITLE: A New Mechanism for Modulating the Activity of the Cancer Invasion Promoter Cyclooxase-2

PRINCIPAL INVESTIGATOR: Parkson Lee-Gau Chong

CONTRACTING ORGANIZATION: Temple University
Philadelphia, PA 19140

REPORT DATE: September 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE	*Form Approved* *OMB No. 0704-0188*

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
30-09-2008	Annual	01 SEP 2007 - 31 AUG 2008

4. TITLE AND SUBTITLE
A New Mechanism for Modulating the Activity of the Cancer Invasion Promoter Cyclooxase-2

5a. CONTRACT NUMBER W81XWH-07-1-0587

5b. GRANT NUMBER BC063769

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Parkson Lee-Gau Chong

Email: pchong02@temple.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Temple University
School of Medicine
3420 North Broad Street
Philadelphia, PA 19140

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

*

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Cyclooxygenase-2 (COX-2) and its product PGE2 are known to promote tumor growth and to enhance the penetration of cancer cells into adjacent tissues. Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The goal is to unravel a new molecular mechanism for regulating the activity of COX-2. In plasma membranes, arachidonic acid (AA) is released by phospholipase A2 (PLA2). COX then converts AA to prostaglandins. The activity of PLA2 is known to vary with cholesterol content in an alternating manner, showing a local minimum at critical sterol mole fractions for maximal superlattice formation. In this study, the cholesterol content in MCF-7 human breast cancer cells was depleted by using methyl-beta-cyclodextrin. A biphasic change in COX-2 activity was observed at certain cell cholesterol content Cr. The cholesterol content near C_r could serve as a fine-tuning mechanism to regulate COX-2 activity and PGE2 production, and consequently, cancer cell growth and metastasis

15. SUBJECT TERMS
Membranes; cyclooxygenase; cancer invasion; MCF-7 cells; cellular signaling

16. SECURITY CLASSIFICATION OF: U			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

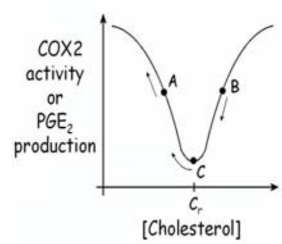
Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

Objectives. Cyclooxygenase-2 (COX2) and its product PGE_2 (a prostaglandin) are known to increase both angiogenesis and resistance to apoptosis (promoting tumor growth) and to enhance the penetration of cancer cells into adjacent tissues (causing metastasis) [1]. Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The **goals** of this proposed research are to unravel a new molecular mechanism for regulating the activity of COX2 and to provide evidence that this mechanism may regulate cell proliferation in cultured breast cancer cells.

Hypothesis. Arachidonic acid (AA) is released from the plasma membrane by phospholipase A2 (PLA2). Cyclooxygenase (COX) converts AA to prostaglandins (e.g., PGE_2). The activity of PLA2 is known to vary with membrane cholesterol content in an alternating manner [2], showing a local minimum at critical sterol mole fractions (C_r) for maximal superlattice formation [3]. Hence, it is logical to **hypothesize** that the activity of COX (including the isoform COX2) also varies with cholesterol content in a biphasic manner, showing a minimum at C_r (see diagram). The biphasic change in COX2 activity should occur within a narrow range of cholesterol content near C_r. As a result, C_r serves as a bio-switch that can regulate the production of PGE_2. When the cholesterol content is near C_r, the COX2 activity and the PGE_2 production are low. When the cholesterol content deviates significantly from C_r (either >> C_r or << C_r), the COX2 activity, thus the production of PGE_2, becomes high. As such, the cholesterol content near C_r serves as a fine-tuning mechanism to regulate COX2 activity and PGE_2 production, and consequently, cancer cell growth and metastasis.



Innovation. This research may reveal a new role of cholesterol in breast cancer. According to this hypothesis, the initial membrane cholesterol content in cells will determine how a decrease in cholesterol content would affect COX2 activity. For example, if the initial membrane sterol content is at A or C, a decrease in cholesterol will bring up the COX2 activity. If the initial sterol content is at B, a decrease in cholesterol will decrease the cellular activity (until it reaches C). This implies that while one could take cholesterol lowering drugs, such as statins, to reduce serum cholesterol and thereby lower cardiovascular risks, the decrease in cholesterol might also greatly increase PGE_2 production, causing an increase in tumor growth and cancer invasion. In fact, animal studies showed that long-term statin use may be carcinogenic [4] and epidemiological evidence suggests that some statins increase the incidence of breast cancer [5].

BODY

Cells. The human MCF-7 breast cancer cells (from ATCC) were grown in Complete Growth Medium which includes Dulbecco's Modification of Eagle's Medium (DMEM 1x, with 4.5g/L glucose, L-glucose & sodium pyruvate) supplemented with 10% Fetal Bovine Serum (FBS, BioWhittaker, Walkersville, MD) and 1% penicillin/streptomycin at 37°C with 5% CO_2 atmosphere. Cell culture prior to confluence were harvested and used for our experiments.

Modification of cell cholesterol content. For cholesterol depletion, cells were incubated in serum-free medium with different amounts of methyl-β-cyclodextrin (MβCD) for various times at 37^{o}C. Following incubation with cells, MβCD was removed by washing with PBS buffer.

COX-2 activity measurements. The activity of exogenously added human recombinant COX-2 was triggered by addition of phospholipase A2 (PLA2) to cells and monitored at 37^{o}C using the COX-2 activity kit from Cayman Chemicals. The COX activity assay kit measures the peroxidase activity of COX. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) at 590 nm using a microplate reader. The kit includes isozyme-specific inhibitors for distinguishing COX-2 activity from COX-1 activity. The COX-2 activity will be calculated using the TMPD extinction coefficient of 0.00826 μM^{-1}. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmole of TMPD per minute at 25^{o}C. It takes 2 molecules of TMPD to reduce PGG_2 to PGH_2. The COX-2 activity was plotted as a function of cholesterol content relative to total membrane lipids in the cell or a function of [MβCD]. Lipids in cells were extracted by a chloroform-methanol solvent mixture (2:1, v/v). The chloroform layer was taken out for cholesterol and phospholipid determinations.

Results.

Figure 1 (see Supporting Data) demonstrates that the COX-2 activity in MCF-7 cells varies with cell cholesterol content in a non-monotonic way, we have carried out the following preliminary experiments. The MCF-7 cells were treated with 0.0, 0.25, 0.75, 1.0, and 1.75% MβCD for one hour. The cells were then washed twice to remove MβCD. Two sets of samples were tested. The results were reproducible and showed a trend of biphasic change in COX-2 activity with cell cholesterol content. The %MβCD is inversely proportional to cell membrane cholesterol. These data provide supporting evidence that the proposed experiment is feasible and that our hypothesis is reasonable and testable. These results suggest that the cholesterol dependence of COX-2 activity is likely to be multi-biphasic.

The detection of multiple biphasic changes at particular sterol mole fractions could be a sign of possible sterol superlattice formation. However, to demonstrate the cholesterol dependence of COX-2 activity is multi-biphasic, we need to extend the study to a larger cholesterol mole fraction range using smaller cholesterol increments. A real challenge is whether small cholesterol increments (e.g., 1 mol%) in cells could be readily created or measured. In a separate study, we were able to reliably create ~1-2 mol% cholesterol increments in Chinese

Hamster Ovary (CHO) cells and demonstrated that the determinations of cholesterol content relative to phospholipids, in most cases, were accurate to 0.2-0.7 mol%. Using these experimental conditions, we were able to demonstrate a biphasic change in lipid raft density isolated from CHO cells at, at least, two cholesterol mole fractions, a sign consistent with the concept of sterol superlattice formation. Thus, we are confident that similar cholesterol mole fraction increments can be achieved in MCF-7 cells. In the no-cost-extension period, we will extend our study of MCF-7 (e.g., the data shown in Figure 1) to a much larger cholesterol range.

Significance. If the hypothesis is correct, then reaching a local minimum in COX2 activity at C_r may be a wise therapeutic goal. **This concept could be used to develop a new treatment strategy for the reduction of breast cancer tumor growth and metastasis, which could then be used in combination with existing treatments.** The idea is that when the membrane cholesterol level deviates appreciably from those values which produce minimum COX2 activity, the cells are more prone to cancer proliferation and invasion due to higher levels of PGE_2 production. In this case, one should manipulate the cholesterol content in cell plasma membrane by locally applying cholesterol lowering or enrichment drugs to the troubled tissues via targeted drug delivery technology.

A list of personnel receiving payments:

Berenice Venegas, Associate Scientist
Michelle Olsher, Associate Scientist
Parkson Chong, PI

KEY RESEARCH ACCOMPLISHMENTS

- COX-2 activity varies strongly with cell cholesterol content.
- COX-2 activity varies with cell membrane cholesterol content in a non-monotonic manner.
- There is a sign that COX-2 activity in MCF-7 breast cancer cells varies with cell cholesterol content in a multiple biphasic manner, in agreement with the concept of sterol superlattice.

REPORTABLE OUTCOMES

Meeting abstracts

Chong, P. L.-G., Olsher, M., Venegas, B., Zhu, W., and Tran, S. (2008) A New Mechanism for Modulating the Activity of the Cancer Invasion Promoter Cyclooxygenase-2. A poster presented at the Era of Hope meeting in Baltimore, Maryland, June, 2008.

Zhu, W., Olsher, M., Venegas, B., Tran, S. and Chong, P. L.-G. (2009) Role of Membrane Cholesterol Content on the Activity of Cyclooxygenase-2 (COX-2) in MCF-7 Human Breast Cancer Cells. A poster to be presented at the Biophysical Society Meeting in Boston, MA, March 2009.

CONCLUSION

We were able to modify the cholesterol content in MCF-7 human breast cancer cells and demonstrate that the COX-2 activity varies with cholesterol content in a non-monotonic manner. This result provides a good starting point to eventually demonstrate that the COX-2 signaling pathway is regulated by the extent of sterol superlattice.

REFERENCES

1. Yiu, G. K., and Toker, A. (2006) NFAT Induces Breast Cancer Cell Invasion by Promoting the Induction of Cyclooxygenase-2. *J. Biol. Chem. 281*, 12210-12217.

2. Liu, F., and Chong, P. L.-G. (1999) Evidence for a Regulatory Role of Cholesterol Superlattices in the Hydrolytic Activity of Secretory Phospholipase A2 in Lipid Membranes. *Biochemistry. 38*, 3867-3873.

3. Somerharju, P., Virtanen, J. A., and Cheng, K. H. (1999) Lateral Organisation of Membrane Lipids. the Superlattice View. *Biochim. Biophys. Acta. 1440*, 32-48.

4. Lam, L., and Cao, Y. (2005) Statins and their Roles in Cancer. *Drugs Today (Barc). 41*, 329-334.

5. Duncan, R. E., El-Sohemy, A., and Archer, M. C. (2005) Statins and Cancer Development. *Cancer Epidemiol. Biomarkers Prev. 14*, 1897-1898.

APPENDICES

Two meeting abstracts (see above)

1. Chong, P. L.-G., Olsher, M., Venegas, B., Zhu, W., and Tran, S. (2008) A New Mechanism for Modulating the Activity of the Cancer Invasion Promoter Cyclooxygenase-2. A poster presented at the Era of Hope meeting in Baltimore, Maryland, June, 2008.

Background and objectives:

Cyclooxygenase-2 (COX2) and its product PGE_2 (a prostaglandin) are known to promote tumor growth by increasing angiogenesis, metastasis and resistance to apoptosis. Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The goals of this proposed research are to unravel a new molecular mechanism for regulating the activity of COX2 and to provide evidence that this mechanism may regulate cell proliferation in cultured breast cancer cells.

Arachidonic acid (AA) is released from the plasma membrane by the action of phospholipase A2 (PLA_2); COX2 then converts AA to prostaglandins. The activity of PLA_2 is known to vary with membrane cholesterol content in a biphasic manner, showing a local minimum at critical sterol mole fractions (C_r) for maximal superlattice formation. We hypothesize that the activity of COX2 will also vary in an alternating manner, allowing plasma membrane cholesterol content to function as a bioswitch to regulate the production of PGE_2. When the cholesterol content is near C_r, the COX2 activity and the PGE_2 production are low. When the cholesterol content deviates significantly from C_r (either >> C_r or << C_r), the COX2 activity, thus the production of PGE_2, becomes high. As such, the cholesterol content near C_r serves as a fine-tuning mechanism to regulate COX2 activity and PGE_2 production, and consequently, cancer cell growth and metastasis.

Description:

Using methyl-beta-cyclodextrin (mβCD), we are altering cholesterol levels in very small increments (~1 mol%) in the plasma membranes of MCF-7 cells. After cholesterol depletion, COX2 activity levels can be measured using the COX2 activity kit (Cayman Chemicals). The COX2 activity will be then plotted as a function of cholesterol content relative to total membrane lipids in the plasma membrane. Total membrane lipids will be determined by capillary electrophoresis and mass spectrometry. The extent of cancer cell proliferation will be determined fluorometrically using a CyQUANT assay kit from Molecular Probes on cells with varying cholesterol content alterations. All the experiments will be performed in triplicate.

Results:

We are currently assessing the results of preliminary COX2 assays. Using methyl-beta-cyclodextrein (mβCD), we are altering the cholesterol levels in the plasma membranes of MCF-7 cells in very small increments over a wide cholesterol concentration range. After cholesterol depletion, COX2 activity levels can be measured in different cell cultures with varying membrane cholesterol concentrations.

Conclusions:

If the hypothesis is correct, then reaching a local minimum in COX2 activity at C_r may be a wise therapeutic goal. This concept could be used to develop a new treatment strategy for the reduction of breast cancer tumor growth and metastasis, which could then be used in combination with existing treatments. The idea is that when the membrane cholesterol level deviates appreciably from those values which produce minimum COX2 activity, cells are more prone to cancer proliferation and invasion due to higher levels of PGE_2 production. In this case, one should manipulate the cholesterol content in cell plasma membrane by locally applying cholesterol lowering or enrichment drugs to the troubled tissues via targeted drug delivery technology.

2. Zhu, W., Olsher, M., Venegas, B., Tran, S. and Chong, P. L.-G. (2009) Role of Membrane Cholesterol Content on the Activity of Cyclooxygenase-2 (COX-2) in MCF-7 Human Breast Cancer Cells. A poster to be presented at the Biophysical Society Meeting in Boston, MA, March 2009.

Cyclooxygenase-2 (COX-2) and its product PGE2 are known to increase both angiogenesis and resistance to apoptosis (promoting tumor growth) and to enhance the penetration of cancer cells into adjacent tissues (causing metastasis). Thus, knowing how the activity of COX2 is regulated at the cellular level has implications for breast cancer therapeutic strategies. The goal of this research is to unravel a new molecular mechanism for regulating the activity of COX-2. The proposed molecular mechanism may be elucidated by using the sterol superlattice model. In plasma membranes, arachidonic acid (AA) is released by phospholipase A2 (PLA2). Cyclooxygenase (COX) then converts AA to prostaglandins (e.g., PGE2). The activity of PLA2 is known to vary with membrane cholesterol content in an alternating manner, showing a local minimum at critical sterol mole fractions (C_r) for maximal superlattice formation. Hence, it is logical to hypothesize that the activity of COX (including the isoform COX-2) also varies with cholesterol content in a biphasic manner. In this study, the cholesterol content in MCF-7 human breast cancer cells was depleted by using methyl-beta-cyclodextrin. A biphasic change in COX-2 activity was observed at certain cell cholesterol content C_{rcell}. The cholesterol content near C_{rcell} could serve as a fine-tuning mechanism to regulate COX-2 activity and PGE2 production, and consequently, cancer cell growth and metastasis.

SUPPORTING DATA

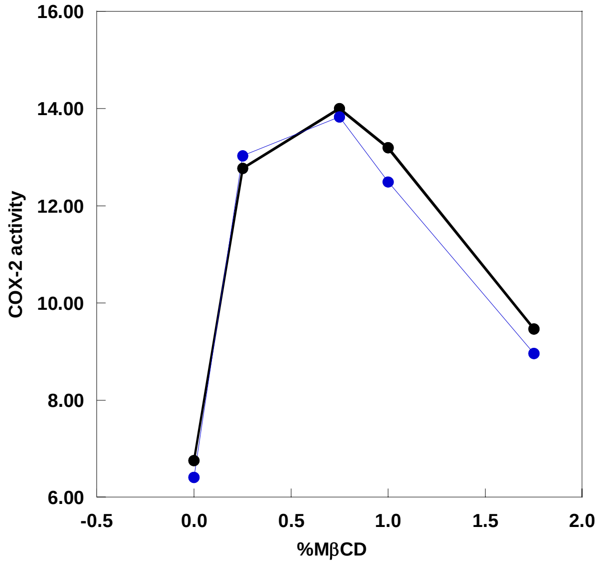


Figure 1. Plot of COX-2 activity versus weight percent of MβCD used to treat MCF-7 cells. Activity was measured at 24°C.